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CERTIFICATE

This certificate is issued in support of an application for Patent registration in a country outside New Zealand pursuant to the Patents Act 1953 and the Regulations thereunder.

I hereby certify that annexed is a true copy of the Provisional Specification as filed on 13 September 2002 with an application for Letters Patent number 521434 made by AgResearch Limited.

Dated 29 September 2003.

Neville Harris
Commissioner of Patents, Trade Marks and Designs

COMMISSIONER OF PATENTS, TRADEMARKS & DESIGNS
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PROVISIONAL SPECIFICATION

NOVEL ALLERGEN

I/WE AGRESEARCH LIMITED a New Zealand Company of East Street, Ruakura Campus, Hamilton, New Zealand

do hereby declare this invention to be described in the following statement:

NOVEL ALLERGEN

TECHNICAL FIELD

The present invention relates to novel allergens. In particular the present invention relates to nucleotide sequences encoding novel allergens of a nematode parasite. The invention broadly concerns allergens obtained from, but by no means exclusively from, *Trichostrongylus colubriformis* and use of the nucleotide and amino acid sequences of the allergens to identify animals that are resistant to nematodes to assist with selective breeding of resistant animals in particular but by no means exclusively nematode infections in sheep.

BACKGROUND TO THE INVENTION

The present invention is preferably but not necessarily exclusively derived from *Trichostrongylus colubriformis* and has application to disinfection of the nematode or similar nematodes as discussed herein. For ease of reference only the present invention will now be described in relation to *Trichostrongylus colubriformis* infection.

Trichostrongylus colubriformis is a nematode parasite which infects the small intestine of sheep and is of significant economic importance in New Zealand and worldwide. These parasites have a simple life cycle consisting of free-living stages on pasture (egg to infective larvae, L3), and after ingestion, develop through L4-L5-adult in the host gastrointestinal tract. They do not have a tissue migratory phase. *T. colubriformis* live in mucus covered tunnels eroded on the surface of duodenal and intestinal villi.

Presently the only effective means of controlling *T. colubriformis* and other nematodes that infect sheep is through the use of anthelmintics.

Sheep can develop natural immunity to *T. colubriformis* following repeated natural infections. Development of immunity to establishment requires at least seven weeks of continuous infection (Dobson et al 1990). Two or three periods of truncated infections

where anthelmintic treatment is used to remove infection 10-14 days later has been used as an effective means of immunising sheep.

However, a major drawback with conventional anthelmintics is that nematode resistance to a broad spectrum of anthelmintics is now becoming increasingly more widespread and is therefore of serious concern. (Waller, 1997; Sangster *et al*, 1999; Van Wyk *et al*, 1999).

Various studies have suggested that immunity to *T. colubriformis* involve the development of Th2-type immune responses involving hypersensitivity type events. These responses include increased mast cell, globule leucocytes and eosinophil numbers in the intestinal mucosa. Elevated serum IgE levels have also been associated with immunity to nematode infections (Shaw et al., 1998a & b). One possible role of parasite-specific IgE in resistance to nematode infection is its involvement in immediate hypersensitivity reactions, where cross-linking of specific IgE on mucosal mast cells by antigen promotes their degranulation, which in sheep results in the formation of globule leukocytes. The resultant release of inflammatory mediators is considered to be a major mechanism involved in the rejection of parasites in immune sheep. The release of inflammatory mediators may cause a deterioration of the intestinal microenvironment and consequent expulsion of nematode parasites. The inflammatory response may also allow the leakage of immunoglobulins into the intestinal lumen and which may also assist in the expulsion of nematodes from the gastrointestinal tract.

Antigens that elicit hypersensitivity reactions by binding to IgE (i.e. allergens) are therefore useful in diagnostic assays to identify immune animals with a Th2-type hypersensitivity immune response. Thus, such antigens are useful in selective breeding programs fro producing animals that are resistant to nematodes.

A major allergen of *Trichostrongylus colubriformis*, Tco-Aspin with a molecular weight of 31 kDa, pI 5.1 on 2-D electrophoresis gels has been identified by the inventors and its nucleotide and polypeptide sequence determined. It shares homology with a number of proteins identified as nematode aspartyl protease inhibitors. From *Onchocerca volvulus* an aspartyl protease inhibitor like protein has been cloned by a number of investigators. Ov33-3 (Lucius, et al., 1988) an immunodominant antigen

recognised by 96% of onchocerciasis patients, Oc3.6 (Chandrashekar, et al., 1991), OvD5B (Celine Nkenfou, Thesis, "Molecular Cloning of Genes Coding Antigens Specific For Onchocerca volvulus: Evaluation of Expressed Proteins For Use In The Diagnosis Of Onchocerciasis" University of Cameroon (1993). Orthologs have been found in *Brugia malayi* (Bm33) (Dissanayake 1993), *Acanthocheilonema viteae* (Av33) (Willenbucher, et al., 1993), *Dirofilaria immitis* (DiT33) (Maja, et al., 1994, Frank et al., 1998), *Parelaphostrongylus tenuis* (Ptpi) (Duffy et al., 2002) and *Ostertagia ostertagi* (Claerebout et al.,2002)..

Some of these have been used successfully to diagnose important filarial infections of humans including *O. volvulus* (Chandrashekar, et al., 1991 & 1996) and *B. malayi* (Chandrashekar, et al., 1994; Dissanayake , et al., 1993).

It is an object of the present invention to address the foregoing problems or at least to provide the public with a useful choice.

Further aspects and advantages of the present invention will become apparent from the ensuing description which is given by way of example only.

All references, including any patents or patent applications, cited in this specification are hereby incorporated by reference. No admission is made that any reference constitutes prior art. The discussion of the references states what their authors assert, and the applicants reserve the right to challenge the accuracy and pertinency of the cited documents. It will be clearly understood that, although a number of prior art publications are referred to herein, this reference does not constitute an admission that any of these documents form part of the common general knowledge in the art, in New Zealand or in any other country.

SUMMARY OF INVENTION

The present invention broadly concerns isolated nucleic acid molecules and polypeptides associated with nematode infection and their use to identify animals that are resistant to nematodes, in particular but by no means exclusively nematode infections in sheep to assist selective breeding of resistant animals. In particular, the invention is directed towards the uses of the aspartyl protease inhibitor (Tco-Aspin), of *Trichostrongylus colubriformis*, in identifying animals that are resistant to nematodes.

According to a first aspect of the present invention there is provided an isolated polypeptide comprising an amino acid sequence as set forth in SEQ ID NO. 1.

According to a second aspect of the present invention there is provided an isolated polypeptide substantially as described above, or a functional fragment or variant thereof, wherein the polypeptide, or fragment or variant thereof, provokes a humoral and/or cellular immunological response in an animal.

According to a third aspect of the present invention substantially as described above wherein the functional fragment or variant incorporates a B cell or T cell epitope of the polypeptide.

In general the polypeptide maybe derived from a nematode parasite of the superfamily Trichostrongyloidea. However, it should also be appreciated that the polypeptide may also be artificially synthesized for example recombinantly synthesized

In particular the nematode parasite may be selected from the genera consisting of *Trichostrongylus, Cooperia, Dictyocaulus, Haemonchus, Ostertagia* or *Teladorsagia.*

Preferably, the nematode parasite maybe of the genera *Trichostrongylus* and is preferably selected from the species consisting of *T. axei or, T. vitrinus* and most preferably is *T. colubriformis.*

In a fourth aspect of the present invention there is provided an isolated nucleic acid molecule wherein the molecule:

a) comprises a nucleotide sequence as set fourth in SEQ ID NO. 2

b) is a functional fragment or variant of the molecule(s) in a); or

c) is able to hybridise under stringent conditions to the molecule(s) in a) or b); or

d) is a complement of the molecule(s) defined in a), b) or c); or

e) is an anti-sense sequence corresponding to any of the sequences in a) – d).

In a fifth aspect of the present invention there is provided a nucleic acid molecule encoding a polypeptide substantially as described above.

In a sixth aspect of the present invention there is provided a vector or construct comprising the nucleic acid molecule of the present invention.

In a seventh aspect of the present invention there is provided a host cell which has been transformed with a vector or construct of the present invention.

In an eighth aspect of the present invention there is provided a ligand which binds to a polypeptide of the present invention.

In a ninth aspect of the present invention there is provided an isolated probe capable of hybridizing under stringent conditions to a nucleic acid molecule of the present invention.

In a tenth aspect of the present invention there is provided a method for determining the immune status of an animal to a nematode infection characterized by steps of:

a) obtaining a blood or serum sample from the animal;

b) preparing an IgE enriched or IgG depleted preparation of the sample in a);

c) contacting the sample at a) with a polypeptide SEQ ID NO. 1;

d) contacting the preparation from c) with a probe;

e) detecting the probe to identify the immune status of the animal by the presence or absence of the probe.

In an eleventh aspect of the present invention there is provided a method for determining the immune status of an animal to a nematode infection characterized by steps of:

a) obtaining a blood or serum sample from the animal

b) preparing an IgE enriched or IgG depleted preparation of the sample in a) using

ammonium sulphate precipitation or affinity purification of sheep IgE;

c) Exposing the preparation from b) with a polypeptide of SEQ ID NO. 2 coated onto microtiter plates;

d) Detection of immuno-complex formed by the polypeptide and IgE at step c) with mouse monoclonal antibodies to ovine IgE.

e) Detection of ovine IgE with appropriately labeled anti mouse antibodies.

Preferably, the sample may be exposed to the polypeptide via an enzyme-linked immunoassay (ELISA) or other suitable type of assay.

In general the animal may be selected from the group consisting of sheep, goats, cattle and equines. However, this list should not be seen as limiting as the animal may be any mammal which is prone to infection with nematode parasites.

Most preferably the animal is a sheep.

It is to be clearly understood that the invention also encompasses peptide analogues, which include but are not limited to the following:

1. Compounds in which one or more amino acids is replaced by its corresponding D-amino acid. The skilled person will be aware that retro-inverso amino acid sequences can be synthesised by standard methods; see for example Chorev and Goodman, 1993;

2. Peptidomimetic compounds, in which the peptide bond is replaced by a structure more resistant to metabolic degradation. See for example Olson *et al*, 1993; and

3. Compounds in which individual amino acids are replaced by analogous structures for example, gem-diaminoalkyl groups or alkylmalonyl groups, with or without modified termini or alkyl, acyl or amine substitutions to modify their charge.

Other uses of the proteins include:

According to a twelfth aspect there is provided a method of determining the immune status of an animal comprising the steps of:

a) exposing a portion of the animal's skin to a polypeptide of the present invention;

b) determining the immune status by the presence or absence of an immune or allergic reaction.

According to a thirteenth aspect there is provided a method for selectively breeding animals resistant to nematode infection characterized by steps of:

a) determining the immune status of male and female animals;

b) selectiving resistant males and females;

c) using selected animals to breed progeny resistant to said infection.

DISCLOSURE OF INVENTION

The present invention is broadly directed to nematode antigens or functional fragments or variants thereof, molecules associated with such, and their use in diagnosing, treating or preventing nematode infection.

The inventors have identified a number of IgE binding antigens (allergens) of *T. colubriformis* using western blotting techniques with purified ovine IgE from various immune animals. Using 2-D electrophoresis, the target allergens have been isolated and identified using mass spectrometry. Recombinant allergen proteins were prepared and tested for their ability to bind IgE and to induce protection of sheep in immunisation trials.

The term "variant" as used herein refers to nucleotide and polypeptide sequences wherein the nucleotide or amino acid sequence exhibits substantially 70% or greater homology with the nucleotide or amino acid sequences contained in the sequence listing, preferably 75% homology and most preferably 90-95% homology to the

sequences of the present invention. – as assessed by GAP or BESTFIT (nucleotides and peptides), or BLASTP (peptides) or BLASTN (nucleotides). The variant may result from modification of the native nucleotide or amino acid sequence by such modifications as insertion, substitution or deletion of one or more nucleotides or amino acids or it may be a naturally-occurring variant.

Thus, the term variant should be taken to include changes (i.e. conservative substitution) to the nucleotide sequences set forth in the sequence which do not alter the amino acid being coded for due to the degenerate nature of the genetic code. The term "variant" also includes homologous sequences which hybridise to the sequences of the invention under standard or preferably stringent conditions.

In general "stringent conditions" for determining the degree of homology may refer to:

a) low salt concentrations (i.e. less than 1M, preferably less than 500mM and most preferably less that 200mM); and

b) high hybridization temperatures (i.e. at least 30°C, preferably greater than 37°C and most preferably greater than 50°C).

However, as the stringency of hybridization can be affected by other factors including probe composition and the presence of organism solvents, it is the combination of parameters above that, which is important in determining stringency.

For example, stringent hybridization conditions can be defined as 2 x SSC at 65°C, or and for example standard hybridization conditions can be defined as 6 x SCC at 55°C, provided always that the variant is capable of diagnosing, treating or preventing nematode infection. Where such a variant is desired, the nucleotide sequence of the native DNA is altered appropriately. This alteration can be effected by synthesis of the DNA or by modification of the native DNA, for example, by site-specific or cassette mutagenesis. Preferably, where portions of cDNA or genomic DNA require sequence modifications, site-specific primer directed mutagenesis is employed, using techniques standard in the art.

The term "isolated" means substantially separated or purified away from contaminating sequences in the cell or organism in which the nucleic acid naturally occurs and

includes nucleic acids purified by standard purification techniques as well as nucleic acids prepared by recombinant technology, including PCR technology, and those chemically synthesised. Preferably, the nucleic acid molecule is derived from genomic DNA or the mRNA of the *Trichostrongylus colubriformis*.

The nucleic acid molecule may be an RNA, cRNA, genomic DNA or cDNA molecule, and may be single- or doublestranded. The nucleic acid molecule may also optionally comprise one or more synthetic, non-natural or altered nucleotide bases, or combinations thereof.

A fragment of a nucleic acid is a portion of the nucleic acid that is less than full length and comprises at least a minimum sequence capable of hybridising specifically with a nucleic acid molecule according to the present invention (or a sequence complementary thereto) under stringent conditions as defined below. A fragment of a polypeptide is a portion of the polypeptide that is less than full length but which still retains a biological function of being capable of diagnosing, treating or preventing nematode infection. A fragment according to the invention has at least one of the biological activities of the nucleic acid or polypeptide of the invention.

The polypeptides of the invention can be prepared in a variety of ways. For example, they can be produced by isolation from a natural source, by synthesis using any suitable known techniques (such as by stepwise, solid phase, synthesis described by Merryfield (1963), *J.Amer.Chem.Soc.* Vol 85:2149-2156) or as preferred, through employing DNA techniques.

The cloning vector may be selected according to the host or host cell to be used. Useful vectors will generally have the following characteristics:

(a) the ability to self-replicate;

(b) the possession of an appropriately positioned single target for any particular restriction endonuclease; and

(c) desirably, carry genes for a readily selectable marker such as antibiotic resistance.

Two major types of vector possessing these characteristics are plasmids and bacterial viruses (bacteriophages or phages). Presently preferred vectors include the bacteriophage lambda Uni-ZAP™ XR and the modified plasmid pBAD18 vector, AY2-4. Tight regulation, modulation, and high-level expression by vectors containing the arabinose PBAD promoter. *J. Bacteriol.* 177:4121-4130).

The DNA molecules of the invention may be expressed by placing them in operable linkage with suitable control sequences in a replicable expression vector. Control sequences may include origins of replication, a promoter, enhancer and transcriptional terminator sequences amongst others. The selection of the control sequence to be included in the expression vector is dependent on the type of host or host cell intended to be used for expressing the DNA.

Generally, eucaryotic, yeast, insect or mammalian cells are useful hosts. Also included within the term hosts are plasmid vectors. Suitable procaryotic hosts include *E. coli*, *Bacillus* species and various species of *Pseudomonas*. Commonly used promoters such as β-lactamase (penicillinase) and lactose (lac) promoter systems are all well known in the art. Any available promoter system compatible with the host of choice can be used. Vectors used in yeast are also available and well known. A suitable example is the 2 micron origin of replication plasmid.

Similarly, vectors for use in mammalian cells are also well known. Such vectors include well known derivatives of SV-40, adenovirus, retrovirus-derived DNA sequences, Herpes simplex viruses, and vectors derived from a combination of plasmid and phage DNA.

Further eucaryotic expression vectors are known in the art (e.g. P.J. Southern and P.Berg, *J. Mol. Appl. Genet.* 1 327-341 (1982); S. Subramani et al., *Mol.Cell.Biol.* 1, 854-864 (1981); R J. Kaufmann and P.A. Sharp, "Amplification and Expression of Sequences Cotransfected with a Modular Dihydrofolate Reducase Complementary DNA Gene, *J. Mol. Biol.* 159, 601-621 (1982); R J. Kaufmann and P.A. Sharp, *Mol.Cell.Biol.* 159, 601-664(1982); S.I. Scahill et al., "Expressions And Characterization Of The Product Of A Human Immune Interferon DNA Gene In Chinese Hamster Ovary Cells," *Proc. Natl. Acad. Sci. USA.* 80, 4654-4659 (1983); G. Urlaub and L.A. Chasin, *Proc. Natl. Acad. Sci. USA.* 77, 4216-4220, (1980).

The expression vectors useful in the present invention contain at least one expression control sequence that is operatively linked to the DNA sequence or fragment to be expressed. The control sequence is inserted in the vector in order to control and to regulate the expression of the cloned DNA sequence. Examples of useful expression control sequences are the lac system, the trp system, the tac system, the trc system, major operator and promoter regions of phage lambda, the glycolytic promoters of yeast acid phosphatase, e.g. Pho5, the promoters of the yeast alpha-mating factors, and promoters derived from polyoma, adenovirus, retrovirus, and simian virus, e.g. the early and late promoters of SV40, and other sequences known to control the expression of genes of prokaryotic and eucaryotic cells and their viruses or combinations thereof.

A preferred promoter for use herein is the arabinose promoter (Guzman, L., Belin, D., Carson, M. J. and Beckwith, J.,1995. Ref), however, any suitable promoter is included within the scope of the present invention as would be appreciated by a skilled worker.

In the construction of a vector it is also an advantage to be able to distinguish the vector incorporating the foreign DNA from unmodified vectors by a convenient and rapid assay. Reporter systems useful in such assays include reporter genes, and other detectable labels which produce measurable colour changes, antibiotic resistance and the like. In one preferred vector, the β-galactosidase reporter gene is used, which gene is detectable by clones exhibiting a blue phenotype on X-gal plates. This facilitates selection. In one embodiment, the β-galactosidase gene may be replaced by a polyhedrin-encoding gene; which gene is detectable by clones exhibiting a white phenotype when stained with X-gal. This blue-white color selection can serve as a useful marker for detecting recombinant vectors.

The term "ligand" refers to any molecule which can bind to another molecule such as a polypeptides or peptide, and should be taken to include, but not be limited to, antibodies, cell surface receptors or phage display molecules.

It should be appreciated that the term "antibody" encompasses fragments or analogues of antibodies which retain the ability to bind to a polypeptide of the invention, including but not limited to Fr, $F(ab)_2$ fragments, ScFv molecules and the like. The

antibody may be polyclonal but is preferably monoclonal. In some embodiments the ligand may be a phage display molecule.

"Primers" are short nucleic acids, preferably DNA oligonucleotides 15 nucleotides or more in length, which are annealed to a complementary target DNA strand by nucleic acid hybridization to form a hybrid between the primer and the target DNA strand, then extended along the target DNA strand by a polymerase, preferably a DNA polymerase. Primer pairs can be used for amplification of a nucleic acid sequence, e.g. by the polymerase chain reaction (PCR) or other nucleic acid amplification methods well known in the art. PCR-primer pairs can be derived from the sequence of a nucleic acid according to the present invention, for example, by using computer programs intended for that purpose such as Primer (Version 0.5© 1991, Whitehead Institute for Biomedical Research, Cambridge, MA).

"Probes" as used herein refers to a ligand which is labelled in some way (for example, radioactively, fluorescently or immunologically), and which are used to find and mark a target polypeptide sequence by binding to the target polypeptide.

The preparation of pharmaceutical compositions including pharmaceutical carriers are well known in the art, and are set out in textbooks such as Remington's Pharmaceutical Sciences, 19th Edition, Mack Publishing Company, Easton, Pennsylvania, USA.

For the purposes of this specification it will be clearly understood that the word "comprising" means "including but not limited to", and that the word "comprises" has a corresponding meaning.

The present invention will now be described in detail, by way of reference only, to the following non-limiting examples.

BRIEF DESCRIPTION OF DRAWINGS

Further aspects of the present invention will become apparent from the following description which is given by way of example only and with reference to the accompanying drawings in which:

Figure 1 shows one-dimensional immunoblot analysis of *T. colubriformis* L3 homogenate proteins probed with time-course IgE samples from sheep during the development of immunity to nematode infection. IgE was purified from serum samples taken at various time-points following weaning of lambs in the field out to 18 months of age. Arrows indicate major antigen bands detected on immunoblots (I- 42 kDa, II- 31-32 kDa, III- 27-31 kDa, IV- 20-21 kDa, V- 2-14 kDa).

Figure 2 shows two-dimensional immunoblot analysis of *T. colubriformis* L3 homogenate proteins. Proteins were subjected to isoelectric focusing (left to right) followed by SDS-PAGE (top to bottom). Immunoblot was probed with affinity purified total IgE antibodies from pooled sera from Immune sheep. Spot corresponding to Tco-Aspin is indicated by a circle and Tco-Aspin breakdown product by a square.

Figure 3 shows two-dimensional gel electrophoresis separation of *T. colubriformis* L3 homogenate total proteins Coomassie blue stained. Spot corresponding to Tco-Aspin is indicated by a circle and Tco-Aspin breakdown product by a square.

Figure 4 shows the alignment of Tco-Aspin with other members of the putative aspartyl protease inhibitor family. Database accession numbers for the previously sequenced members are: (Oo) *Ostertagia ostertagi* (CAD10783), (Pt) *Parelaphostrongylus tenuis* (AAG50205), (Ce) *Caenorhabditis elegans* (AAC46663), (Av) *Acanthocheilonema viteae* (S23229), (Di) *Dirofilaria immitis* (AAA70419), (Ov) *Onchocerca* volvulus (AAA29419). Alignment begins at the putative initiating methionines.

Figure 5 shows one dimensional immunoblot of *T. colubriformis* adult homogenate (lane 1) and L3 homogenate (lane 2) immunostained with rabbit anti Tco-Aspin sera.

Figure 6 shows two-dimensional immunoblot analysis of *T. colubriformis* L3 homogenate proteins. Proteins were subjected to isoelectric focusing (left to right) followed by SDS-PAGE (top to bottom). Immunoblot was probed with serum from rabbit immunized with purified recombinant Tco-Aspin.

Figure 7 shows one-dimensional SDS-Page gel of affinity purified native Tco-Aspin stained with silver stain and corresponding immunoblot developed with rabbit anti Tco-Aspin sera.

Figure 8 shows one-dimensional SDS-Page gel transferred to PVDF of affinity purified native Tco-Aspin stained with Coomassie blue with segments excised for Edman sequencing (A) and corresponding immunoblot developed with rabbit anti Tco-Aspin sera (B)

EXPERIMENTAL

EXAMPLE 1

Nematode larvae and antigen preparation

Infective larvae (3rd stage) of *T. colubriformis* (TcL3) were obtained from cultures of faeces taken from monospecifically infected Romney sheep. Somatic antigen (TcL3-Homog) was prepared by homogenising exsheathed larvae under liquid nitrogen in a mortar and pestle. Soluble protein was extracted in 5 mM Tris buffer pH 7.6 with protease inhibitor (Complete, Boehringer Mannheim) added. After centrifugation at 3000 rpm for 10 min, the protein concentration of the supernatant was determined by absorbance at 230/260 nm, before being aliquoted and frozen at -70°C.

EXAMPLE 2

Two-dimensional gel electrophoresis

Sample preparation and isoelectric focusing was performed as described in the manufacturer's instruction manual with slight modifications. Briefly, 900 µl of TcL3-Homog (~360-1920 µg) was precipitated in 3600 µl acetone at -20°C for 30-120 min. After centrifuging at 17000g for 30 min at 4°C, acetone was removed and the precipitate dried. The precipitate was dissolved in ~280 µl of rehydration buffer containing 8 M urea, 3 M thiourea, 4% CHAPS, 40 mM dithiothreitol, 0.5% IPG buffer (pH 3-10 or pH 4-7) and a trace of Bromophenol blue. The solution was used to rehydrate Immobiline DryStrips (13 or 18 cm, pH 3-10 or pH 4-7) at 30V for 30-60 hr on an IPGphor (Amersham Pharmacia Biotech). Proteins were focused at 20°C according to the following voltage protocol: 120V for 2 hr, 500V for 1hr, 1000V for 1 hr, 1000-8000V gradient for 30 min, 8000V for 6.0-8.5 hr (Total Vhr were 52000 to 80000 Vhr). After a standard equilibration step, proteins were run in the second dimension on 10 to 18% linear SDS-Page gels. Proteins in the resolved gels were either stained with Copper (Bio-Rad) or microwave assisted Coomassie blue R-250 (Wong 2000) or transferred to nitrocellulose according to the manufacturer's directions (Bio-Rad).

EXAMPLE 3

Western blotting

Proteins transferred to nitrocellulose were initially detected with Ponceau S (Harper & Speicher, 1995) and the membrane marked to assist later identification of proteins in gels for further analysis. All incubations were at room temperature and washing of membranes was with PBS + 0.05% Tween 20, 3 x 5 min. Following blocking with Blotto, nitrocellulose was probed with purified sheep IgE (Shaw et al., 1997) at 10-15 µg ml^{-1} overnight. IgE was obtained from sheep infected monospecifically with T *colubriformis* or field grazing sheep and which showed high levels of immunity as determined by faecal egg count and worm counts (data not shown). Membranes were incubated sequentially with mouse monoclonal anti-ovine IgE antibodies (XB6 & YD3 1/15 dilution of culture supernatant) and horseradish peroxidase-conjugated goat anti-mouse IgG gamma chain specific (1/1000) (Sigma Chemical) before detection with 3-Amino-9-ethylcarbozole. A protein spot corresponding to a strong IgE binding spot on

Western blots with molecular weight 33,000 and pI 5.1 (Tco-Aspin) was analysed to determine amino acid sequence information.

EXAMPLE 4

Protein in-gel digestion

Protein spots from Coomassie blue stained gels were identified by comparison with IgE stained companion Western blots. Spots were excised, destained and digested with trypsin (Shevchenko et al., 1996). Briefly, spots were destained in 25 mM NH_4HCO_3 in 50% acetonitrile (ACN). Spots were then dried by centrifugal evaporation and rehydrated in 25mM NH_4HCO_3 containing 12.5 μg ml-l trypsin and incubated at 37°C for 16 hr. Peptides were then extracted sequentially with 25 mM NH_4HCO_3, 50% ACN/0.5% trifluoroacetic acid (TFA)(3 times) and 100% ACN. The combined extracts were dried in a Speedvac, rinsed with milliQ water, then dried again. Extracts were then dissolved in 0.5%TFA. The tryptic digest was cleaned up with ZipTips (Millipore) according to the manufacturer's directions. Peptides were eluted from ZipTips in matrix solution consisting of a saturated solution of α-Cyano-4-hydroxycinnamic acid in 50% ACN/ 0.5% TFA and spotting directly onto Maldi sample plate.

EXAMPLE 5

Protein identification by peptide mass fingerprinting

Molecular masses of tryptic peptides from each protein spot were determined on a MALDI-tof instrument equipped with a nitrogen laser at 337 nm (Perceptive Biosystems, Voyager mass spectrometer). All MALDI spectra were externally calibrated using CALMIX 2. Peptide masses were submitted for protein mass database searching at ProFound (URL: http://www.proteometrics.com/prowl-cgi/ProFound.exe).

EXAMPLE 6

De novo peptide sequencing by Electrospray ionization

Protein spots were submitted to the Australian Proteome Analysis Facility (APAF, Sydney, Australia) for determination of amino acid sequence from selected peptides. Briefly samples undergo a 16-hour tryptic digest at 37°C. The resulting peptides were purified using a ZipTip to concentrate and desalt the sample. The samples were then analysed by ESI-TOF MS/MS using a Micromass Q-TOF MS equipped with a nanospray source, using either flow injection coupled to a Waters CapLC, or manually acquired using borosilicate capillaries for nanospray acquisition. Data was acquired over the m/z range 400-1800Da to select peptides for MS/MS analysis. After peptides were selected, the MS was switched to MS/MS mode and data collected over the m/z range 50-2000Da with variable collision energy settings. Amino acid sequences were then determined from this data.

EXAMPLE 7

N-terminal amino acid sequencing

Crude T. colubriformis L3 protein was separated by 2-D electrophoresis (as in EXAMPLE 2) and electroblotted to PVDF membrane using standard techniques for preparing proteins for sequencing. The PVDF was stained with 0.1% Ponceau S and or 0.025% Coomassie blue R the spots corresponding to allergens on companion Western blots (EXAMPLE 3) were identified and cut out. PVDF spots were then submitted for Edman degradation analysis to establish the amino acid sequence of the amino terminus for as many residues as could be established.

EXAMPLE 8

Molecular Biology

The protein spot when subjected to QTOF mass spectrometry yielded the sequence of two peptides, TC41A and TC41I. Also the mature amino terminus of the protein was determined by protein sequencing. Forward and reverse degenerate oligonucleotides were designed and used as primers in polymerase chain reaction (PCR) under

permissive conditions. *T. colubriformis* total RNA was prepared with Trizol (GibcoBRL) using manufacturers protocols except the initial extraction was performed by grinding larvae in presence of Trizol under liquid Nitrogen. Total RNA was converted to cDNA by standard procedures using SuperscriptII (GibcoBRL) and used as the template in subsequent PCR. The primers used in PCR are listed below.

SL1 GGTTTAATTACCAAGTTTGA

Tco-Aspin TC41Afor GAACAGCAGGAAATCACCAAYTTAYGARAA

Tco-Aspin TC41Arev TTTTCGTAGTTGGTGATTTCYTGYTGYTC

Tco-Aspin TC41Ifor GGAGARGCTGARCARTT

Tco-Aspin TC41Irev AAYTGYTCAGCYTCNCC

TC41.sig.5.nde GATCGGCGCGCCATATGGCACCAAGACAGAACGC

Tco-Aspin TC41.not.3 GATCTGCGGCCGCATAGATCCTCGTGCAGAAGTT

PolyTA TTTTTTTTTTTTTTTTTTTTA

PolyTC TTTTTTTTTTTTTTTTTTTTC

PolyTG TTTTTTTTTTTTTTTTTTTTG

PCR reactions were performed with *T. colubriformis* cDNA with various combinations of these primers, in addition to the nematode spliced leader primer (SL1). PCR conditions using degenerate primers was as follows; 95° for 2', followed by 10 cycles of 95° for 30sec, 35° for 45sec, + 1.0° per cycle, 72° for 45sec followed by 30 cycles of 95° for 30sec, 45° for 45sec, + 0.2° per cycle, 72° for 60sec, + 2sec per cycle. A product of 240bp was observed by polyacrylamide gel electrophoresis of products following reactions with the primer combination of SL1 and TC41Arev while all other combinations yielded no detectable PCR product. PCR products were cloned into pCR2.1 using TA cloning (Invitrogen) and sequenced using ABI 377 automated DNA sequencer (Waikato DNA sequencing facility). Analysis of the sequence revealed homology to the 5' end of various nematode protease (pepsin) inhibitor mRNAs. It was also noted that a previously identified EST clone (unpublished) had homology to these

protease (pepsin) inhibitors at the 3' end. This allowed the synthesis of Tco-Aspin specific 5' and 3' primers, TC41.not3 and TC41.sig.5.nde, each containing restriction endonuclease sites for cloning into the expression vector AY2-4. These primers were used to generate the mature Tco-Aspin coding sequence in a PCR reaction using *T. colubriformis* cDNA as template. After PCR the products were restricted with the appropriate restriction enzyme and gel purified (Qiagen). The resultant DNA products were digested b appropriate restriction enzymes, cloned into the expression vector AY2-4 and the cloned insert was sequenced.

Bacteria containing the Tco-Aspin/ AY2-4 construct were grown in LB broth at 37°C to an optical density (600nm) of 0.8 at which time protein synthesis was induced by the addition of 0.2% L(+)arabinose (BDH). Induction proceeded for 16 hr at 30 at which time the induced bacteria were pelleted and resuspended in 300mM NaCl/50mM $P0_4$ pH 8.0 buffer containing 1mg/ml lysozyme and incubated on ice for 1 hr. The bacteria were sonicated and the solution clarified by centrifugation to yield a soluble bacterial fraction. Recombinant protein was immobilized to Ni-NTA resin (Qiagen), washed with 300mM NaCl/50mM $P0_4$ pH 8.0 buffer containing 20mM imidazole to remove the contaminating *E. coli* proteins after which the recombinant protein was eluted by the addition of 100mM imidazole. The recombinant protein was subsequently dialyzed versus 300mM NaCl/50mM $P0_4$ pH 8.0 buffer to remove the imidazole and the protein concentration determined. Recombinant Tco-Aspin was also purified from the insoluble, inclusion body fraction. In this case the bacterial pellet following centrifugation of the lysed bacteria was solubilised in 8M urea, 300mM NaCl/50mM $P0_4$ pH 8.0 buffer and applied to the Ni-NTA column equilibrated in the same buffer. After washing with several column volumes, the column was washed in the same buffer without urea prior to elution in imidazole as above.

EXAMPLE 9

Immunisation of Rabbits

New Zealand white rabbits were immunized with purified 6-his tagged recombinant protein. Immunizing doses consisting of 100 µg of Tco-Aspin mixed with Montanide ISA50 at a ratio of 6 parts Montanide: 5 parts aqueous solution were injected intra muscularly and subcutaneously. Four weeks later a second immunizing dose prepared

exactly as the first, was administered by the same route. The rabbit was bled by heart puncture under anesthetic 10 days after the second immunization. After clotting for 1 hour at room temperature the blood was centrifuged at 1300g for 15 minutes. The serum was collected and stored at –20°C until needed. This serum is referred to as anti Tco-Aspin.

EXAMPLE 10

Affinity purification of native Tco-Aspin

Rabbit anti Tco-Aspin IgG was purified from sera by Protein G sepharose affinity using standard techniques. Purified antibody was bound to NHS-activated sepharose (Amersham Pharmacia) as per the manufacturer's protocols. TcL3-homog was passed through the immobilised rabbit antibody column at 0.5-2.0 ml/min. The column was washed with buffer (20 mM phosphate buffer, 500 mM NaCl, pH 7.0) until a baseline was reached at absorbance of 280 nm. The bound native Tco-Aspin was eluted with either 0.1M glycine or formic acid pH 3.0. The elutant was neutralised with 1M Tris or ammonium hydrogen carbonate pH 8.0. Eluted fractions were vacuumed dried before analysis by 1-D electrophoresis.

EXAMPLE 11

Enzyme immunoassay for specific IgE and IgG_1 to recombinant proteins

Antigen-specific IgG1 and IgE were detected by ELISA as described previously (Shaw et al 1998a). Optimal concentration of soluble recombinant Tco-Aspin having the amino acid sequence SEQ ID NO. 1 were determined to be 0.2 and 5 $\mu g\ ml^{-1}$ in PBS for IgE and IgG1 assays respectively. To get an indication of antibody levels in field sheep, serum samples were obtained from the AgResearch's selectively bred Romney sheep lines selected for high (susceptible) or low (resistant) faecal nematode egg count. Samples were also taken at the same time from an unselected control flock. Results were expressed as mean absorbance in O.D. units. For statistical analysis antibody O.D values were $\log_e$ transformed to normalise the distributions.

Specific IgE immunoassay method:

Serum samples were pre-treated by precipitating 500 µl of serum with 500 µl of 76% saturated ammonium sulphate in distilled water. Samples were vortexed for 10 sec, repeated at 15 min, and then after 30 min centrifuged in a microcentrifuge (10 min at 13,000 rpm). The supernatant was collected and diluted 1:1 in distilled water plus 0.1% Tween 20 in preparation for assay. Wells of microtiter plates (Costar EIA/RIA plate (#9017), Corning Incorporated, Corning, NY, USA) were coated for 2 hours at 37°C with optimally diluted recombinant Tco-Aspin (0.2µg ml^{-1}, 100 µl) in PBS pH 7.2. Following 3 washes with 0.05% (v/v) Tween 20 in distilled water (water-T20), plates were blocked for 5 min in 5% skim milk powder in PBS. Plates were washed 6 times in water-T20. Microtiter plates were then incubated with precipitated serum supernatant (100 µl per well in duplicate) overnight at room temperature. Following 6 washes in PBS-Tween 20, plates were incubated sequentially with mouse monoclonal antibody to ovine IgE (1/15 culture supernatant XB3/YD3, Shaw et al, 1998), goat anti-mouse gamma chain specific peroxidase conjugate (Sigma, St. Louis, Mo, USA). The enzyme substrate 3,3'5,5' tetramethylbenzidine was added and incubated for 30 min. The reaction was stopped 2N H_2SO_4. Plates were read using a 450 nm filter.

EXAMPLE 12

Skin Testing

Sheep were injected intradermally at either of two sites, the relatively wool-free area of the inside thighs, or flank (after clipping of wool). Native or recombinant Tco-Aspin (0.1-10 µg) in PBS was injected using 27 gauge needles. Wheal and flare reactions were observed and measured approximately 30 min after injection.

Results

To identify allergenic proteins, TcL3-homogenate was separated electrophoretically, electroblotted and immunostained with affinity purified ovine IgE. Previous studies using 1-D SDS-Page gels had identified protein bands at approximate molecule weights of 12-14, 20-21, 27-31, 31-32 and 42 kDa as being associated with immunity to *T. colubriformis* infections in field grazing sheep (Figure 1). Using 2-D

electrophoresis technology, a protein spot corresponding to a strong IgE binding spot on Western blots with molecular weight 33,000 and pI 5.1 (Tco-Aspin) (Figure 2) was cut out from Coomassie blue stained 2-D gel (Figure 3) and analysed to determine amino acid sequence information.

No positive match to known proteins was obtained using peptide mass fingerprinting with peptide masses obtained from 33kDa/pI 5.1 spot. De novo peptide sequencing was possible from 2 peptides obtained from submitted spot. These are; TC41A; 884.9 $[M+2H]^{2+}$ SASEQQE[L/I]TNYEK and TC41I;737.46 $[M+3K]^{3+}$ GEAEQFL. N-terminal sequence obtained using Edman degradation on spots cut out from 2-D gels transferred to PVDF was LTVGTI.

Preparation of recombinant Aspartyl protease inhibitor (Tco-Aspin)

Forward and reverse degenerate oligonucleotide primers were designed from the internal peptide sequences and used in PCR in combination with each other or with primers corresponding to the nematode splice leader sequence and polyT. A PCR product from *T. colubriformis* cDNA was obtained using the primers TC41Arev and SL1. This product was TA cloned into the plasmid pCR2.1 and the insert was sequenced. Analysis of the sequence revealed that this cDNA was from the same gene as a partial cDNA clone previously sequenced from a *T. colubriformis* cDNA library (unpublished). The primers TC41.sig.5.nde and TC41.not.3 were synthesized based on DNA sequences at the amino- and carboxyl terminus of the Tco-Aspin coding region. These primers were used in a PCR with a *T. colubriformis* cDNA template to obtain a complete copy of the coding DNA for Tco-Aspin. The resultant PCR product was sequenced and cloned into the expression vector AY2-4 containing the arabinose promoter, a polyHis domain, and a monoclonal epitope tag. The plasmid was transfected into *E. coli* bacteria and successful transfectants were identified by ampicillin resistance. The recombinant bacteria were grown in culture and induced for expression of Tco-Aspin with arabinose. Recombinant Tco-Aspin was found in both the soluble protein fraction and in the insoluble, inclusion body fraction following induction. The soluble Tco-Aspin was directly purified by immobilised metal affinity chromatography (IMAC). The insoluble fraction was solubilised in a chaotropic agent and then purified by IMAC.

Aspartyl protease inhibitor (Tco-Aspin)

This cDNA sequence of 684 base pairs (SEQ ID NO. 2) codes for a protein of 228 amino acids (SEQ ID NO. 1) with a predicted molecular mass of 25,414 Da and a calculated pI of 5.31. The N-terminus amino acid sequence contains a putative signal peptide (SignalP V1.1; http://www.cbs.dtu.dk/services/SignalP/index.html) with a suggested cleavage site between residues 15 and 16 (alanine-alanine). N-terminal sequencing showed that the mature protein's N-terminus is LTVGTI suggesting that further processing removes the amino acids APRQKR. The putative mature protein of 215 amino acids, without the signal sequence and amino acids APRQKR, would have a predicted molecular mass of 23056.77 Da and pI of 4.93. The sequence does not contain N-glycosylation sites.

Computer searches with the predicted amino acid sequence revealed significant homologies with aspartyl protease inhibitors of *Ostertagia ostertagi* (CAD10783): (% identity/% similarity) (86%/90%), *Parelaphostrongylus tenuis* (AAG50205): (71%/83%), *Caenorhabditis elegans* (AAC46663): (50%/68%), *Acanthocheilonema viteae* (S23229): (40%/59%), *Dirofilaria immitis* (AAA70419): (42%/57%), Onchocerca volvulus (AAA29419): (42%/60%). A Clustal sequence alignment of Tco-Aspin with other members of the putative aspartyl protease inhibitor family is shown in Figure 4. These proteins share more distant homology to a known aspartyl protease inhibitor from the intestinal nematode *Ascaris suum* (Martzen et al, 1990).

Rabbit antiserum to recombinant Tco-Aspin was applied to 1-D blots of TcL3 and Tc-adult homogenate (Figure 5). This identified strong staining bands in TcL3 homogenate at 31 and 22 kDa. Considerably weaker bands of the same molecular weights were seen on the adult homogenate. When rabbit antiserum produced to Tco-Aspin was applied to a 2-D blot of TcL3 homogenate a series of closely placed spots of 32.2-34.5 kDa and pI 4.9-5.7 (Figure 6) were identified. These correspond in localisation to the original spot submitted for amino acid sequencing. A spot at 58.8 kDa, pI 5.6 is thought to be non-specific binding. A further spot at 22.9 kDa, pI 5.7 was stained with rabbit anti Tco-Aspin antiserum and considered to be a breakdown product of Tco-Aspin.

Affinity purified native Tco-Aspin isolated from crude TcL3-homogenate and run on 1-D SDS-page gels (Figure 7) ran as 2 bands of molecular weight 31.3 and 21.8 kDa.

The 22 kDa antigen identified by probing of a 2-D blot of crude TcL3-homogenate and affinity purified from the same material is probably a breakdown product of Tco-Aspin. Attempts to identify this spot (see Figures 3 & 4: square) by amino acid sequencing techniques failed to produce a result, due to insufficient amount of protein in gels. An abundant neighbouring protein positioned below the 22 kDa Tco-Aspin breakdown product on 2-D gels was submitted for amino acid sequencing. Following sequencing of the *T. colubriformis* DNA coding for this protein, it was shown as having homology to a *Caenorhabditis elegans* hypothetical protein Y5F2A.1. No significant IgE binding could be demonstrated to a recombinant version of this protein. Using affinity purified native Tco-Aspin, approximately 40 μg was concentrated before loading on to a 1-D SDS-page gel. The gel was transferred to PVDF and stained to identify bands of Tco-Aspin and its breakdown products (Figure 8). Along with the 31 and 22 kDa protein bands, a third low molecular weight was also identified. These bands were excised and submitted for N-terminal sequencing. Edman sequencing revealed that each protein band had the same N-terminal amino acid sequence of LTVGTI. This shows that these proteins are in fact breakdown products of Tco-Aspin and that the N-terminal end of Tco-Aspin is not degraded. Thus we conclude that the 20-21 kDa band identified by screening of sheep developing immunity to nematode infections (Figure 1) is a breakdown product of Tco-Aspin.

In developing an ELISA for Tco-Aspin, maximal reactivity with purified IgE from immune sheep was achieved with either soluble Tco-Aspin, or after reduction with both soluble and insoluble forms. Optimal protein concentration for coating microtitre plates was found to be 0.2 μg ml^{-1} for specific IgE assays and 2.5 μg ml^{-1} for specific IgG_1 assays using soluble Tco-Aspin.

Immunoreactivity of selection line sheep toward recombinant allergen

Antibody (specific IgE and IgG_1) responses to recombinant Tco-Aspin in selectively bred Romney selection line sheep receiving field infections are shown in Table 1.

Table 1

Mean specific antibody O.D. values (back-transformed) for Wallaceville Selection Line male lambs born 2001.

Sheep Line Type	Tco-Aspin IgE Jan	Tco-Aspin IgE Mar	Tco-Aspin IgG1 Jan	Tco-Aspin Mar	Number of sheep
Susceptible	0.001[a]	0.008[a]	0.559[b]	0.300[a]	37
Controls	0.022[a]	0.053[a]	0.641[NS]	0.471[NS]	49
Resistant	0.200	0.350	0.688	0.529	51

Significantly different from Resistant line sheep

[a] – $P<0.001$

[b] – $P<0.05$

[NS] – Not Significant

Resistant line lambs had significantly higher specific IgE to Tco-Aspin O.D values than susceptible or control lambs in serum samples taken in January and March when the lambs were approximately four and six months of age. In contrast specific IgG1 O.D. values for the same samplings were only significantly different between resistant and susceptible lines of sheep. These results show that specific IgE reactivity to the nematode allergen Tco-Aspin could be used to identify sheep with low nematode faecal egg counts at an age of approximately 4 months.

From the O.D. values above for the Resistant and Susceptible Lines of Romney sheep, it would be expected that a negative genetic correlation would be found between Tco-Aspin (IgE) and log-transformed faecal egg count. This was indeed observed (see

Table 2). For example, the genetic correlation of Tco-Aspin$_{January}$ (IgE) with $\log_e$(FEC$_{January}$ +100) was -0.35 (s.e. 0.15; $P < 0.05$), and with $\log_e$(FEC$_{March}$ +100) was -0.52 (s.e. 0.15; $P < 0.001$). The genetic correlation indicates the degree to which genes controlling the expression of one performance measure in an animal are also associated with the expression of another performance measure. In this case, log FEC and log Tco-Aspin IgE are negatively correlated genetically, so that genetic selection to reduce FEC will be expected to result in increased log Tco-Aspin IgE . This indeed is what was observed in practice in the Resistant line, compared with the Susceptible line sheep.

Table 2. Genetic Correlations between measured antibody traits and $\log_e$ FEC

	Genetic correlation with:	
	$\log_e$(FEC$_{Jan}$+100)	$\log_e$(FEC$_{Mar}$ + 100)
$\log_e$(Tco-Aspin IgE: Jan)	-0.3521 s.e. 0.1489	-0.5228 s.e. 0.1549
$\log_e$(Tco-Aspin IgE: March)	-0.3365 s.e. 0.1300	-0.4752 s.e. 0.2056
$\log_e$(Tco-Aspin IgG1: Jan)	0.0310 s.e. 0.1396	-0.1166 s.e. 0.1454
$\log_e$(Tco-Aspin IgG1: March)	-0.4154 s.e. 0.2528	-0.3137 s.e. 0.2036

Aspects of the present invention have been described by way of example only and it should be appreciated that modifications and additions may be made thereto without departing from the scope thereof.

AGRESEARCH LIMITED

by their Attorneys

JAMES & WELLS

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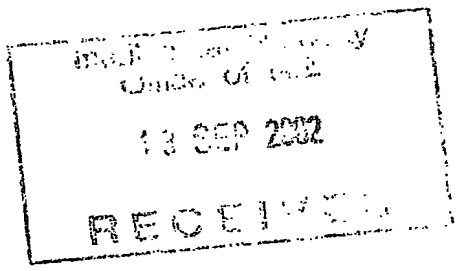

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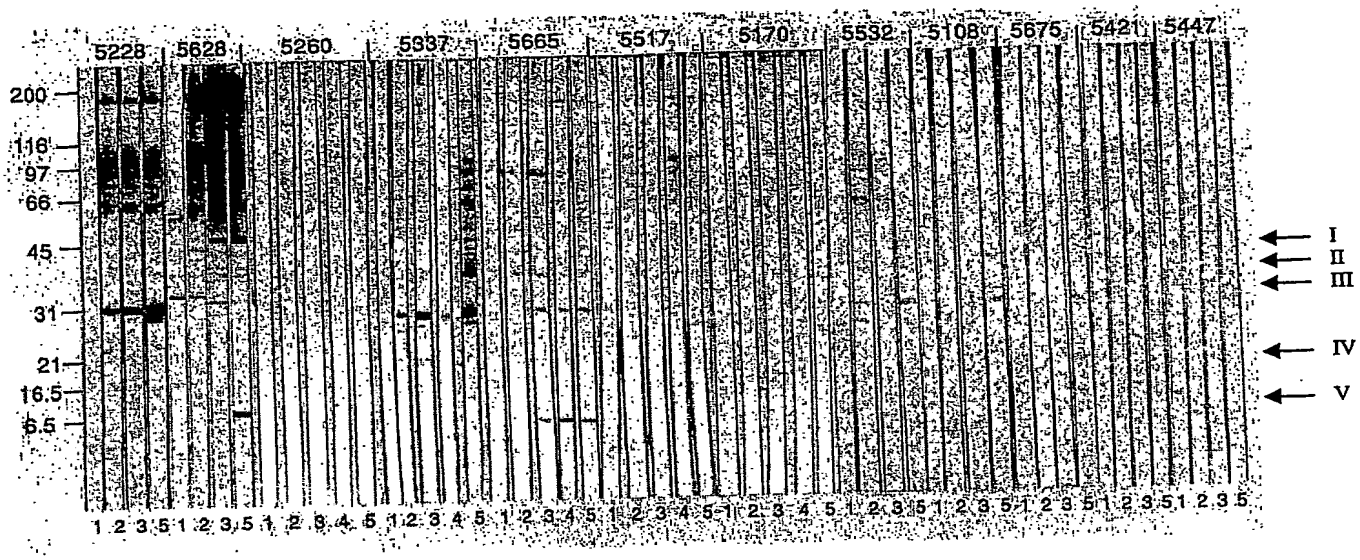
Figure 1.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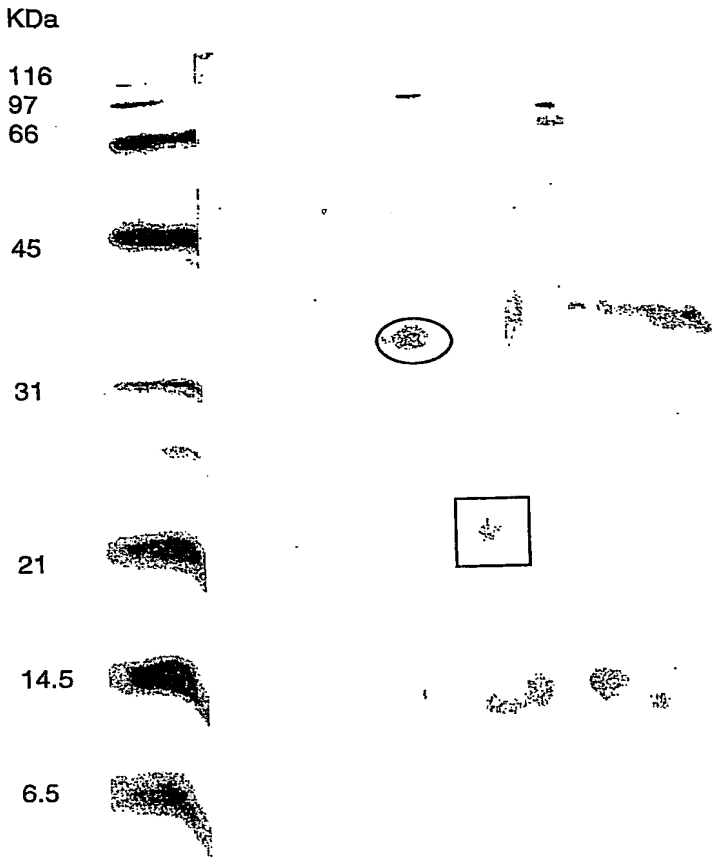

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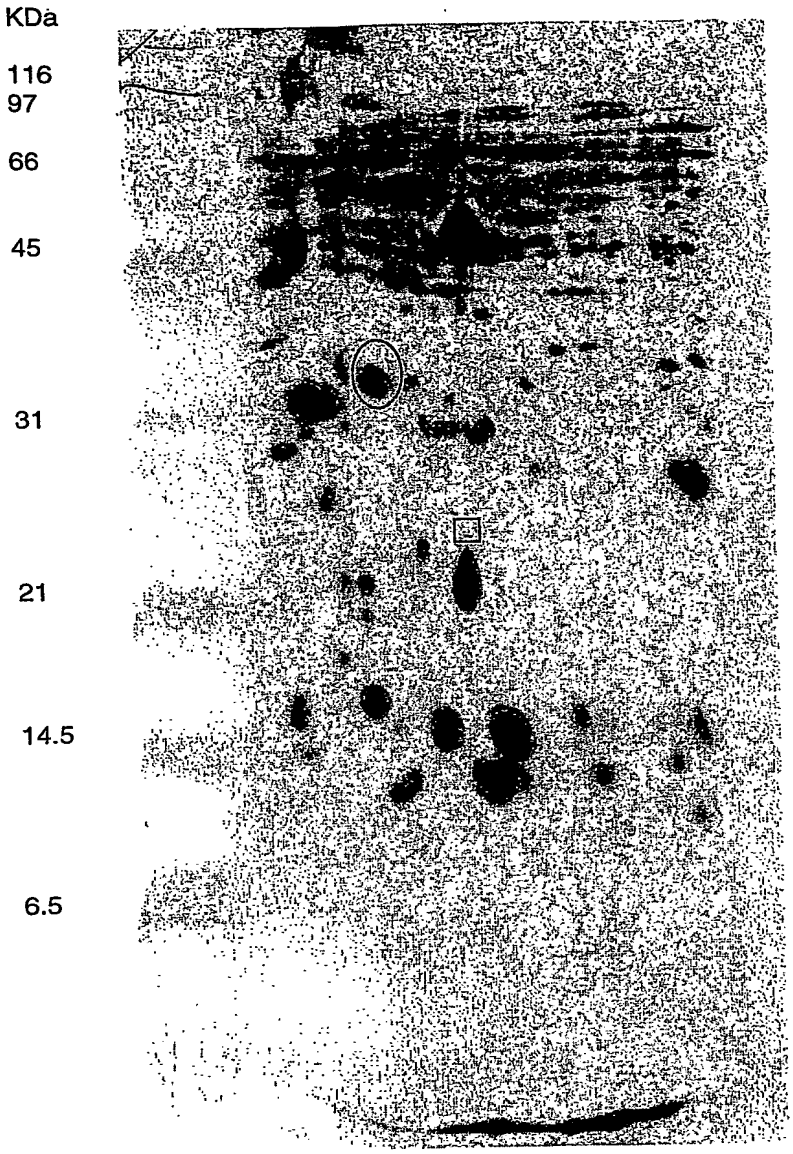
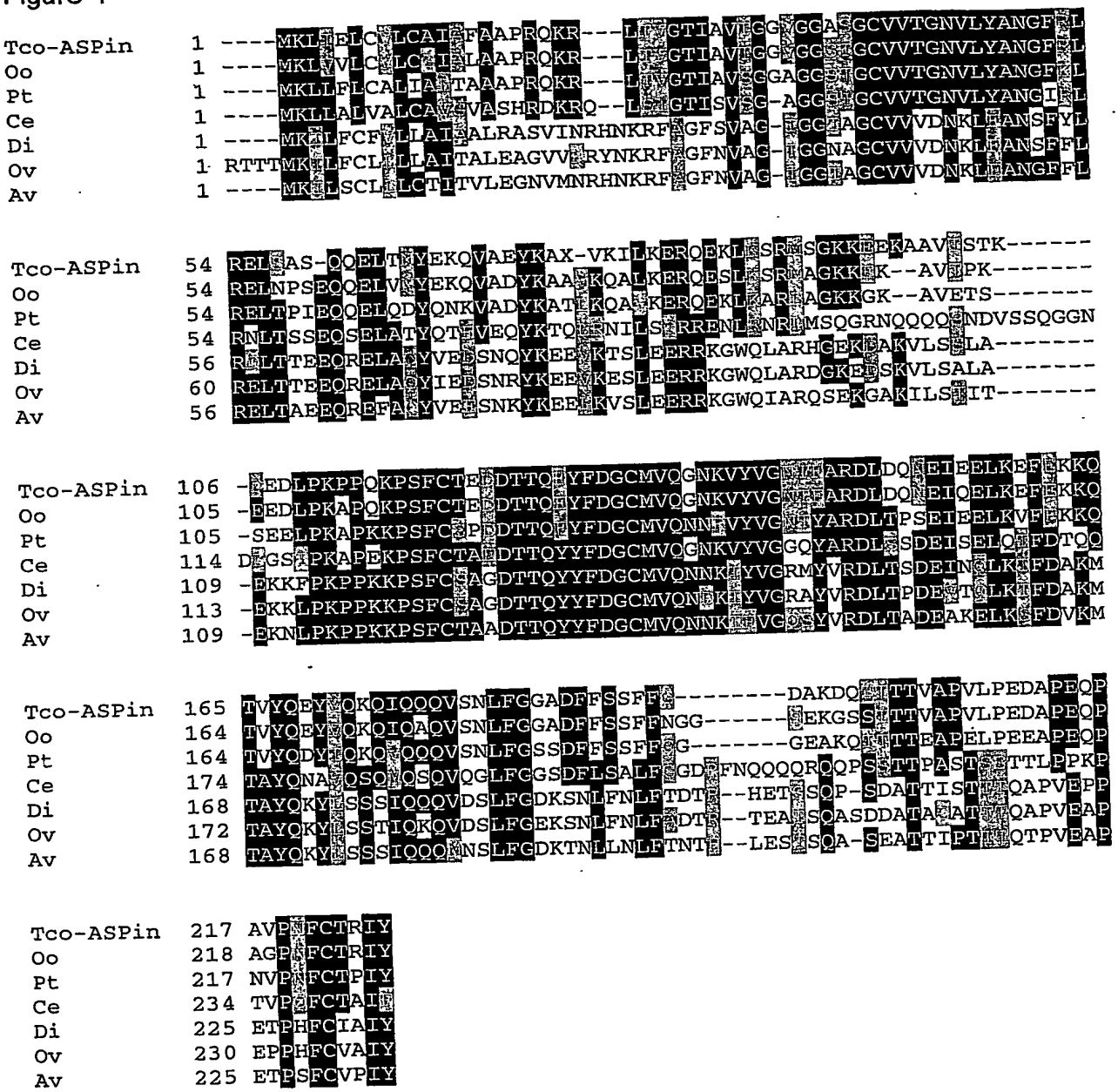


Figure 4



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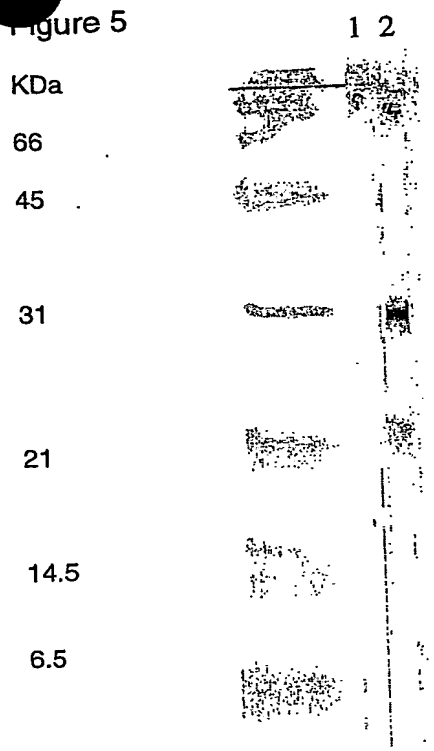

Figure 6

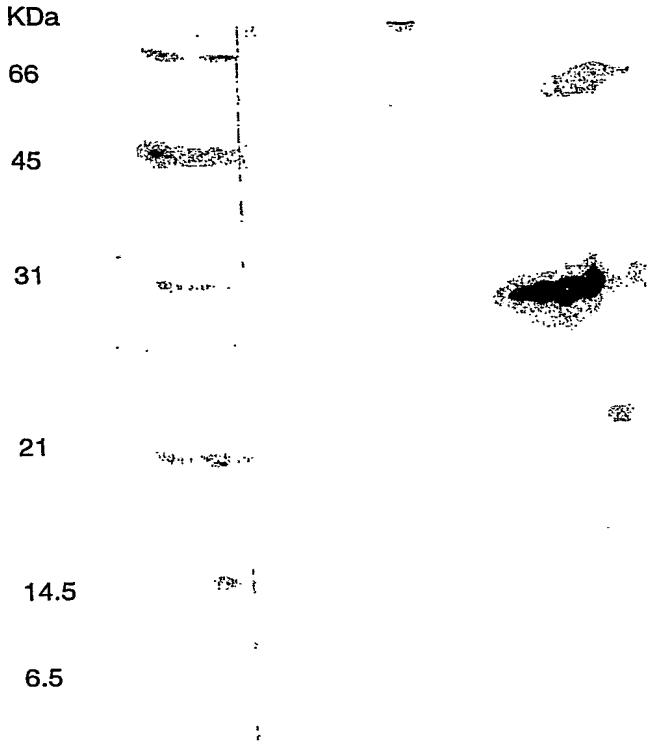

Figure 7

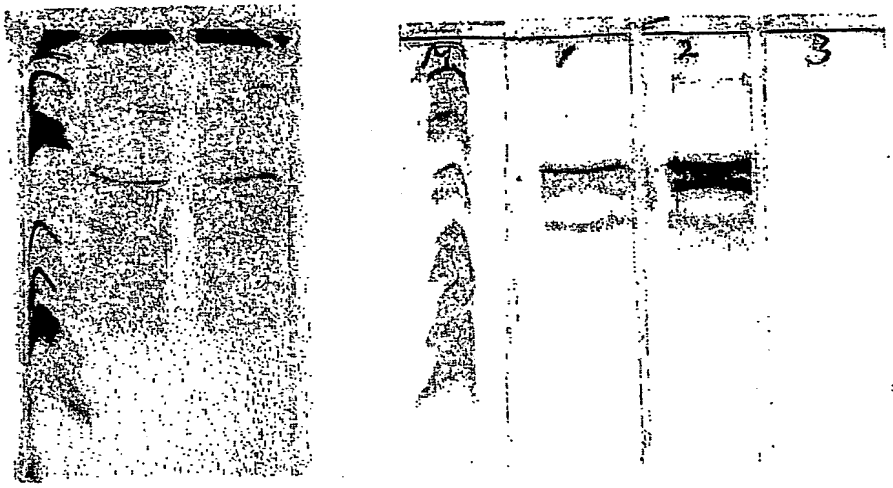

Figure 8